Journal of Forestry Research (2014) 25(3): 535–540
DOI 10.1007/s11676-014-0492-8

ORIGINAL PAPER

Effects of plant growth regulators, carbon sources and pH values on callus induction in *Aquilaria malaccensis* leaf explants and characteristics of the resultant calli

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Received: 2013-01-11; Accepted: 2013-03-18

Abstract: The endangered tropical tree, *Aquilaria malaccensis*, produces agarwood for use in fragrance and medicines. Efforts are currently underway to produce valuable agarwood compoundsn tissue culture. The purpose of this study was to develop an optimal growth medium, specifically, the best hormone combination for callus suspension culture. Using nursery-grown *A. malaccensis*, sterilized leaf explants were first incubated on basic Murashige and Skoog (MS) gel medium containing 15g/L sucrose and at pH 5.7. Different auxin types including 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and indole-3-butyric acid (IBA), were tested at various concentrations (0.55, 1.1 and 1.65 μM) using the basic medium. Leaf explants were incubated for 30 days in the dark. Callus induced by 1.1 μM NAA had the highest biomass dry weight (DW) of 17.3 mg; however the callus was of a compact type. This auxin concentration was then combined with either 6-benzylaminopurine (BAP) or kinetin at 0.55, 1.1, 2.2 or 3.3 μM to induce growth of friable callus. The 1.1μM NAA + 2.2μM BAP combination produced friable callus with the highest biomass (93.3mg DW). When testing the different carbon sources and pHs, sucrose at 15g/L and pH at 5.7 yielded highest biomasses at 87.7mg and 83mg DW, respectively. Microscopic observations revealed the arrangement of the friable cells as loosely packed with relatively large cells, while for the compact callus, the cells were small and densely packed. We concluded that MS medium containing 15 g/L sucrose, 1.1 μM NAA + 2.2 μM BAP hormone combination, and a pH of 5.7 was highly effective for inducing friable callus from leaf explants of *A. malaccensis* for the purpose of establishing cell suspension culture.

Keywords: Agarwood, endangered tree, *in vitro*, plant growth regulators, *Thymelaeaceae*

Project funding: This work was supported by the Universiti Putra Malaysia Research University Grant Scheme (Project No. 03-02-11-1370RU and 03-03-11-1438RU).

The online version is available at http://www.link.springer.com

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Corresponding editor: Chai Ruihai

Introduction

Aquilaria malaccensis is a tropical tree, locally known as "karas." It is the main source of agarwood in Malaysia. The tree occurs in the rainforests of Indonesia, Thailand, Cambodia, Laos, Malaysia, Northern India, Philipines and Borneo (Dash et al. 2008). Agarwood has been highly recognized for its numerous medicinal values and is widely used in perfumery and religious purposes (Naef 2011). *Aquilaria* produces highly valuable resin due to wounding and attack from biological agents such as microbes and insects. High demand for the resin has driven extensive exploitation; the species is now covered under the IUCN Red List Categories, and included in *The World List of Threatened Trees* (IUCN 2011). Studies on other trees and plants showed that wounding triggers the defense system that synthesizes secondary metabolites, in particular terpenes, phenolics, and alkaloids (Bonaventure and Baldwin 2010). Indeed, agarwood oils are high in sesquiterpenes, phenolics, and chromones (Naef 2011)

Plant tissue culture is a system of growing plant cells, tissues, or organs that have been separated from the mother plant in artificial medium under aseptic conditions (Omamor et al. 2007). Tissue culture has largely been integrated into biotechnology as a research tool for callus formation, proliferation, and regeneration of plantlets. Callus is a wound tissue composed of highly vacuo-

lated, unorganized cells. It develops in response to chemical or physical lesions, under determinate hormonal conditions. Calli can be obtained from a tissue fragment and have the ability to differentiate into tissues, organs and even embryos that are able to regenerate into whole plants (Lima et al. 2008).

The exogenous supply of growth regulators is frequently necessary in calogenesis (Nurazah et al. 2009). This necessity refers to the type and concentration of auxin or cytokinin, genotype of the donor plant, and the endogenous content of hormones. Combination of auxins and cytokinins are known to promote cellular differentiation and also organogenesis (Lima et al. 2008). Among the growth regulators used in callus induction, 2,4- dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA), 6-benzylaminnopurine (BAP), and thidiazuron (TDZ) are the most important.

Different growth habits or patterns often influence the characteristics of the resultant calli. Rapid cell division can lead to the formation of a soft friable callus, which is composed of undifferentiated and loosely attached cells. For compact callus, the cells are densely packed and do not favor rapid cell division. Friability of callus tissue is highly desirable when establishing cell suspension culture (Akaneme and Eneobong 2008). A special feature of higher plants is their capacity to produce a large number of secondary metabolites even in cell culture condition (Dash et al. 2008; Rosli et al. 2009). Cell suspension culture could be used to produce these bioactive compounds in a large-scale plant cell culture (Okudera and Ito 2009), which later can be extracted and used in the pharmaceutical, flavor, and fragrance industries. Indeed, plant tissue cultures are found to have potential as supplements to traditional agriculture in the industrial production of desirable bioactive plant metabolites (Vanisree et al. 2004).

This study was aimed at determining the optimal combinations of plant growth regulators, sugar types, and pH for growth of *A. malaccensis* callus culture. In addition, the structure of the callus formed was investigated through histological analysis. These findings provide some basic information on tissue-culture medium suitable for *A. malaccensis*.

Materials and methods

Plant materials

Four- to five-year-old *A.malaccensis* trees were maintained in pot culture with an overhead sprinkler watering system in the shade house of the Faculty of Forestry, Universiti Putra Malaysia, Serdang, Selangor. The third-leaf explants from the apex were collected from healthy lateral branches.

Tissue culture medium

The Murashige and Skoog (MS) medium (Sigma Aldrich, USA) was used at full strength as the basal medium. Sucrose at 15g/L was added into the mixture. The pH of the medium was adjusted to 5.7±0.1 with 1M HCl (AnalaR Normapur®) or 1M KOH (Merck) followed by the addition of 2.75g/L of gelrite (Duchefa, Netherlands). The medium was then autoclaved at 121 °C, 15 psi for 20 min. After autoclaving, a total of 25 mL of sterile medium was poured into 90 mm 15 mm Petri dish in the laminar air flow and allowed to solidify.

The method for surface sterilization of explants was as described previously (Daud et al. 2012). For testing single auxins, leaf explants were cultured on MS medium as prepared above, and supplemented with various types of auxins, including 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and indole-3-butyric acid (IBA) at 0.55, 1.1 and 1.65 μM. Auxin that yielded the highest callus biomass was further combined with a cytokinin. The cytokinins tested were 6-benzylaminopurine (BAP), and kinetin, at 0.55, 1.1, 2.2 and 3.3 μM. All hormones were purchased from Sigma Aldrich, USA. Cultures were maintained completely in the dark in the culture room at 25±1°C. Cultures were incubated for 30 days and daily observations were made to monitor callus growth.

Different sources of carbons such as sucrose, fructose, and glucose—each at 15, 20, 25 and 30 g/L—were also tested to determine their effect on callus growth. The pH of the medium was adjusted at 5.0, 5.5, 5.7, and 6.0 using either KOH or HCl prior to autoclaving. Callus growth was determined by fresh (FW) and dry weight (DW) measurements. For dry weight, callus was dried at 50 °C for 24 hours. Callus growth was also scored on the degree of callus formation and morphology, based on naked eye observation.

Histological analysis

For histological examination, callus was fixed in 10% buffered formalin for 24 hours following the method of Luna (1960). Then, the callus was dehydrated in a series of graded alcohol (80, 95, and 100%) for 1 hour at each percentage and embedded in paraffin wax. Samples were cut into 4 mm sections and were stained with Harri's Hematoxylin and Eosin Staining solutions. The sections were then mounted on Depex (D.P.X) and photographed using Nikon Light Microscope (Japan).

Statistical analysis

All the above experiments were independently repeated three times (each with 30 explants) under the same conditions. Data were analyzed using Proc GLM (SAS version 9.2). Analysis of variance (ANOVA) followed by Duncan's multiple range test for mean comparison at (p =0.05) was used to test statistical significance.

Results

Manipulation of plant-growth regulators is essential to optimize callus induction. In this study, the effect of different concentrations of auxins (2,4-D, NAA and IBA) on callus induction was investigated. After one week in the callus induction medium, the explants started to swell. For those growing in the MS basal

medium without auxin (control), no callus was induced even after 30 days, indicating that plant-growth regulators are required for callus induction. For the explants grown in MS medium with single auxin hormone, the highest biomass of compact callus was obtained from leaf explants grown on MS + 1.1 μM NAA (DW=17.3mg) (Table 1). When using different auxins in single auxin treatment, the explants did not form callus as profusely as when 1.1 μM NAA was used. When combining cytokinin with the best auxin treatment (1.1 μM NAA), BAP at 2.2 μM produced profuse amount of whitish and friable callus (DW=93.3mg) (Table 2). When kinetin was used instead, the amount of biomass recorded was almost half that of the best BAP (2.2 μM) (Table 2).

Table 1. Callus induction from leaf explants of *A. malaccensis* after 30 days in MS medium supplemented with different concentrations of auxins.

Auxins	Concentration (μM)	Degree of callus formation	Color and morphology of callus	Callus DW (mg ± SE)
No auxin	0	NC	NC	NC
2,4-D	0.55	+	Whitish, compact	8.0±0.6^{d,e}
	1.1	++	Whitish, compact	11.3±0.9^{b,c}
	1.65	++	Whitish, compact	12.0±1.5^{b}
NAA	0.55	+	Whitish, compact	9.7±0.7^{c,d}
	1.1	+++	Whitish, compact	17.3±1.8^{a}
	1.65	++	Whitish, compact	13.3±0.9^{b}
IBA	0.55	+	Whitish yellow, compact	8.0±0.6^{d,e}
	1.1	+	Whitish yellow, compact	5.3±0.7^{e}
	1.65	+	Whitish yellow, compact	8.7±1.3^{c,d}

NC = no callus formed, + = very few, ++ = moderate, +++ = profuse. Means followed by the same letters are not significantly different from each other at (p=0.05) determined by Duncan's Multiple Range Test (DMRT). DW= Dry weight, SE=standard error.

Table 2. Callus induction from leaf explants of *A. malaccensis* after 30 days in MS medium supplemented with 1.1 μM NAA and different concentrations of cytokinins.

Concentration (μM)			Degree of callus formation	Color and morphology of callus	Callus DW (mg ± SE)
NAA	BAP	Kinetin			
0.0	0.0	0.0	NC	NC	NC
1.1	0.55	0.0	++	Pale whitish, friable	63.3±2.0^{c}
1.1	1.1	0.0	++	Pale whitish, friable	72.7±2.2^{b}
1.1	2.2	0.0	+++	Pale whitish, friable	93.3±2.0^{a}
1.1	3.3	0.0	++	Pale whitish, friable	62.7±2.2^{c}
1.1	0.0	0.55	+	Whitish yellow, friable	52.3±1.2^{d}
1.1	0.0	1.1	+	Whitish yellow, friable	53.7±1.8^{d}
1.1	0.0	2.2	+	Whitish yellow, friable	54.3±2.0^{d}
1.1	0.0	3.3	+	Whitish yellow, friable	52.7±1.5^{d}

NC = no callus formed, + = very few, ++= moderate, +++ = profuse. Means followed by the same letters are not significantly different from each other at (p=0.05) determined by Duncan's Multiple Range Test (DMRT). DW= Dry weight, SE=standard error.

Next, the effects of different carbon sources at various levels were tested, using the best hormone combination media. Sucrose was found to be the most effective carbon source for inducing callus as it yielded the highest biomass, compared to fructose and glucose (Table 3). Sucrose, at 15g/L significantly induced callus proliferation (DW= 88 mg), yielding a biomass DW of 2-fold and 1.2-fold higher than when fructose and glucose was used, respectively. Higher concentrations of sucrose seemed to yield lower yields (Table 3). Callus growth is much affected by the medium's pH. Callus grown on MS + 1.1 μM NAA + 2.2 μM BAP at pH 5.7 yielded significantly a higher amount of callus (DW=83mg) when compared to callus grown on the same medium at pH 5.0, 5.5 and 6 (Table 4).

Table 3. Callus induction from leaf explants of *A. malaccensis* after 30 days in MS medium supplemented with 1.1 μM NAA + 2.2 μM BAP and different concentrations of carbon sources.

Carbon Source	Concentration (g/L)	Degree of callus formation	Color and morphology of callus	Callus DW (mg ± SE)
Sucrose	15	+++	Pale whitish, friable	87.7±0.9^{a}
	20	++	Pale whitish, friable	75.0±2.5^{b}
	25	++	Pale whitish, friable	75.3±0.9^{b}
	30	++	Pale whitish, friable	70.3±0.9^{c}
Fructose	15	+	Brownish yellow, friable	31.3±0.9^{h}
	20	+	Brownish yellow, friable	41.7±1.2^{g}
	25	+	Brownish yellow, friable	47.3±1.2^{f}
	30	+	Brownish yellow, friable	38.0±0.6^{g}
Glucose	15	+	Whitish, friable	62.7±1.6^{e}
	20	+	Whitish, friable	66.0±1.5^{d,e}
	25	++	Whitish, friable	69.3±1.2^{c,d}
	30	++	Whitish, friable	64.3±2.0^{e}

NC = no callus formed, + = very few, ++= moderate, +++ = profuse. Means followed by the same letters are not significantly different from each other at (p=0.05) determined by Duncan's Multiple Range Test (DMRT). DW= Dry weight, SE=standard error.

Table 4. Effects of different pH values on callus culture from leaf explants of *A. malaccensis* after 30 days in MS medium supplemented with 1.1 μM NAA + 2.2 μM BAP.

pH value	Degree of callus formation	Color and morphology of callus	Callus DW (mg ± SE)
5.0	++	Pale whitish, friable	61.3±0.9^{c}
5.5	++	Pale whitish, friable	76.3±1.5^{b}
5.7	+++	Pale whitish, friable	83.0±2.5^{a}
6	++	Pale whitish, friable	71.3±0.9^{b}

NC = no callus formed, + = very few, ++= moderate, +++ = profuse. Means followed by the same letters are not significantly different from each other at (p=0.05) determined by Duncan's Multiple Range Test (DMRT). DW= Dry weight, SE=standard error.

The study on biomass profile, based on fresh weight (FW) in the callus culture, revealed a typical sigmodal growth curve. The accumulation of biomass in the cultured callus in MS + 1.1 μM

NAA + 2.2 μM BAP yielded the highest amount of biomass (1.99 g/culture FW), 25 days from the first day of culturing (Fig. 1). From the start to the end of day 10, the growth curve revealed that there was no increase in the amount of cells in the culture, indicating that the culture was going through a lag phase. Cell growth increased gradually from day 10 to day 20 as the culture moved into the exponential growth phase (0.86 g/culture FW), after which it entered the stationary phase for about 10 days before necrosis. There was a reduction of biomass profile on day 35 (1.09 g/culture FW). Concurrently, the callus started to turn brown.

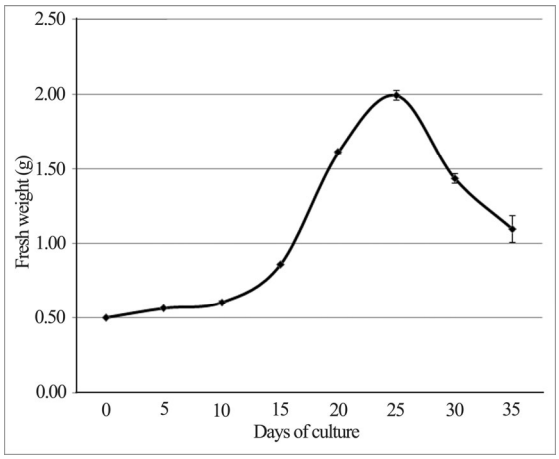

Fig. 1: The growth curve measurement of *A. malaccensis* leaf-derived callus on MS medium supplemented with combination of 1.1 μM NAA + 2.2 μM BAP and incubated at 25°C in completely dark condition. Growth was determined every 5 days after inoculation. Error bars indicate the standard error of mean (n=3).

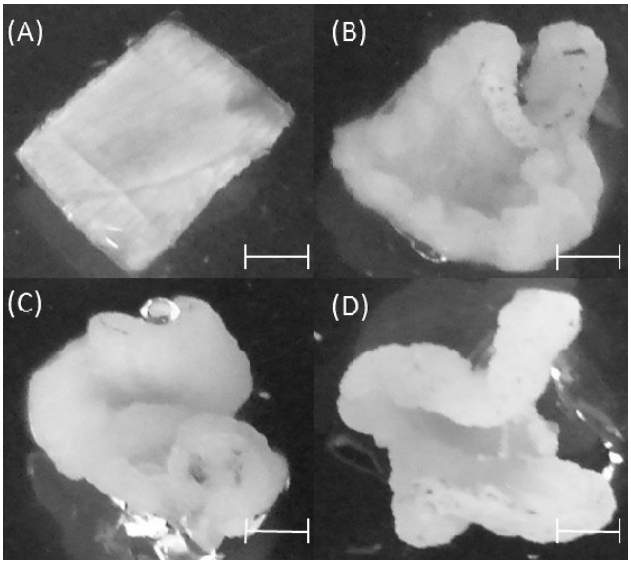

Fig. 2: Callus induction from leaf explants of *A. malaccensis* after 30 days in MS medium supplemented with different concentrations of NAA. Each callus in the photograph is a representative of 30 calli from the same treatment. (A) Control; (B) 0.55 μM; (C) 1.1 μM; (D) 1.65 μM. Bar = 5mm

Finally, the morphology of the callus obtained was distinctly different for every treatment. Compact callus was observed on MS medium supplemented with single auxin, NAA, at various concentrations (Fig. 2). Further investigation on callus morphology showed that when the cytokinin BAP was added into the single auxin medium, friable callus was produced (Fig. 3). The friable callus was soft to touch, watery and brittle, while the compact callus was hard to touch and required some pressure to break it into pieces. We found that MS supplemented with single auxin, NAA at 1.1 μM, produced the highest biomass for compact callus, while the addition of 2.2 μM BAP to this medium yielded callus of the friable type. Microscopic images provided better understanding of the different cell arrangements for friable (loosely packed – Fig. 4a) and compact (closely packed – Fig. 4b) calli.

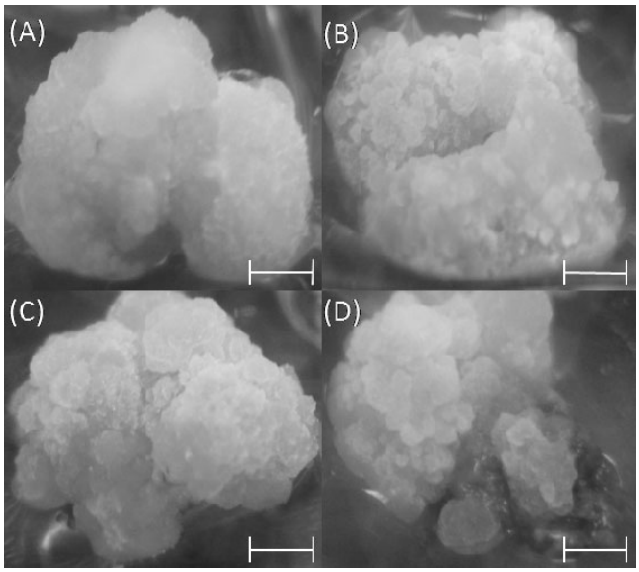

Fig. 3: Callus induction from leaf explants of *A. malaccensis* after 30 days of culture in MS medium supplemented with 1.1μM NAA and different concentrations of BAP. Each callus in the photograph is a representative of 30 calli from the same treatment. (A) 0.55 μM; (B) 1.1 μM; (C) 2.2 μM; (D) 3.3 μM. Bar = 5mm

Discussion

The use of plant growth regulators is of fundamental importance in directing the organogenic response of any plant tissue or organ under *in vitro* conditions (Che et al. 2002; Sugiyama and Imamura 2006). Several studies had been reported regarding the effects of plant growth regulators on callus growth of different plant species (Ling et al. 2013). Combination of plant growth regulators had successfully induced the formation of callus culture from many higher plants (Li et al. 1999; Nurazah et al. 2009). Previous work showed that BAP in combination with NAA and 2,4-D are effective for callus induction using root and leaf explants from *Aquilaria sinensis* (Shu et al. 2005; Okudera and Ito 2009). However, there was no mention of the types of the resultant calli. We found that compact and friable types of calli could be induced from leaf explants of *A.malaccensis* when us-

ing NAA singly or in combination with BAP hormone, respectively.

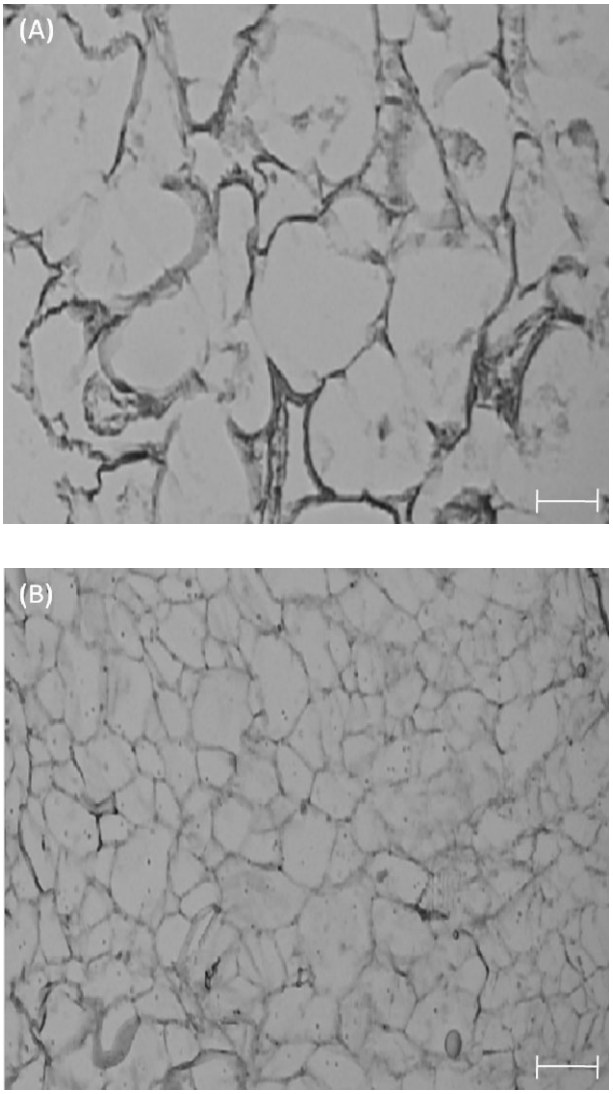

Fig. 4: Microscopic observations on calli induced from leaf explants of *A. malaccensis*. (A) Friable callus derived from combination of 1.1 μM NAA + 2.2 μM BAP showing loosely packed cell mass. (B) Compact callus initiated from single auxin, 1.1 μM NAA, showing compact cell mass. Bar = 5mm

Besides plant growth regulators, sucrose plays an important role by supplying the energy for *in vitro* plant tissue cultures as these have insufficient autotrophic ability. Sucrose not only acts as an external energy source but also contributes to the osmotic potential of the medium (Nowak et al. 2004). These would permit the absorption of mineral nutrients present in the medium, essential to the cells' growth. The significant effect of carbon source concentration (in culture media) on the frequency of callus formation has been noticed in many plants like rice (Shahnewaj and Bari 2004) and olives (Gracia et al. 2002).

However, other types of sugars have also been reported as being suitable carbon sources for tissue cultures of different genera. For example, glucose was found as the best carbon source for callus induction in *Prunus persica* when compared to sucrose and fructose (Declerk and Korban 1996). The pH of the medium is extremely important as it influences the uptake of nutrients and plant growth regulators by regulating their solubility in the culture medium (Bhatia and Ashwath 2005). Plant tissue cultures are known to tolerate a wide range of pHs; values between 5.2 to 5.8 are most often provided (Sanavy and Moeini 2003). Therefore these two factors were carefully tested in our experiments.

The growth of callus culture consists of the lag, exponential, stationary, and death phases (Collin and Edwards 1998). In this study, the growth curve of callus derived from *A.malaccensis* was determined by fresh weight measurement, after optimizing the plant growth regulators and medium composition. It appeared to follow the general growth curve trend. A significant decline in FW happened on day 35 in addition to changes in callus color. The accumulation of toxic products and depletion of nutrients might have led to cell death and eventually a decline in the biomass growth (Karam et al. 2003).

By changing the composition of the media—such as plant growth regulators, carbon sources, and pH values—it is possible to improve tissue dissociation (Akaneme and Eneobong 2008). There are some structural differences between friable and compact calli: microscopic observation revealed that compact callus has cells with small intercellular space, while friable callus has highly vacuolated cells (Na et al. 2007). This is similar to our own observation.

The loosely arranged nature of friable callus may have been the reason for its greater water-retention capacity compared to compact callus (Akaneme and Eneobong 2008). Clearly, friable callus is more desirable than compact callus for growth of cell-suspension culture, where mechanical agitation leads to dispersion of the tissue; compact callus grows as lumps and does not provide suspension (Collin and Edwards 1998).

In conclusion, *A.malaccensis* leaf-derived callus was successfully induced on MS medium with many combinations of plant growth regulators, carbon sources, and pHs; the most effective being 15 g/L sucrose supplemented with 1.1 μM NAA and 2.2 μM BAP at pH 5.7. This medium, particularly the hormone combination, produced friable and pale callus. These findings provide some basic information on the morphology and development of friable callus for establishing cell cultures.

Acknowledgement

This work was supported by the Universiti Putra Malaysia Research University Grant Scheme (Project No. 03-02-11-1370RU and 03-03-11-1438RU).

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